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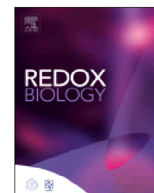

Research Paper

Sequestration of polyunsaturated fatty acids in membrane phospholipids of *Caenorhabditis elegans* dauer larva attenuates eicosanoid biosynthesis for prolonged survival

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A B S T R A C T

Mechanistic basis governing the extreme longevity and developmental quiescence of dauer juvenile, a "non-ageing" developmental variant of *Caenorhabditis elegans*, has remained largely obscure. Using a lipidomic approach comprising multiple reaction monitoring transitions specific to distinct fatty acyl moieties, we demonstrated that in comparison to other developmental stages, the membrane phospholipids of dauer larva contain a unique enrichment of polyunsaturated fatty acids (PUFAs). Esterified PUFAs in phospholipids exhibited temporal accumulation throughout the course of dauer endurance, followed by sharp reductions prior to termination of diapause. Reductions in esterified PUFAs were accompanied by concomitant increases in unbound PUFAs, as well as their corresponding downstream oxidized derivatives (i.e. eicosanoids). Global phospholipidomics has unveiled that PUFA sequestration in membrane phospholipids denotes an essential aspect of dauer dormancy, principally via suppression of eicosanoid production; and a failure to upkeep membrane lipid homeostasis is associated with termination of dauer endurance.

1. Introduction

Survival in nature is characterized by constant episodes of feasts and famines. As such, a number of organisms has evolved developmental variants to survive prolonged period of harsh environmental conditions [1]. The *C. elegans* dauers represent a developmental variant specifically adapted to endure adverse environmental conditions (e.g. food scarcity, overcrowding) for extended duration. Dauers arrest feeding, possess thickened cuticles, often remain motionless, and ration the use of its lipid reserves across the entire period of dormancy, which on average lasts for approximately 30 days for the wild type Bristol N2 strain at 25 °C [2]. The dauer stage is perceived as "non-ageing" [3,4], as dauer entry allows *C. elegans* to significantly "outlive" its normal lifespan (about two weeks) [4]; and that the dauer larva can progress normally into adult stage with unaltered lifespan when favourable conditions return.

Dauer response and survival has garnered considerable research interest, since it is mediated by common pathways that control general metabolism, aging, as well as development, such as the insulin and TGF-β pathways, that are conserved in both *C. elegans* and higher eukaryotes [3]. Indeed, a spatiotemporal compartmentalization of signaling pathways regulating both dauer arrest and normative aging has been previously demonstrated in *C. elegans* [5]. Thus, an in-depth understanding into the mechanistic basis of dauer survival may reveal novel insights to retarding tissue ageing and lifespan extension. Indeed, previous research has demonstrated that dauer entry is mediated by small molecule signals of immense structural diversities, comprising modular assemblies of carbohydrates, amino acids, lipids, as well as fatty acids collectively termed ascarosides [6]. While it is apparent that ascaroside biosynthesis per se serves to integrate nutritional inputs from major metabolic pathways, elucidation of signal perception and integration leading to dauer formation and survival has remained largely obscure, primarily owing to the structural complexities of ascarosides as well as the plethora of associated downstream phenotypes [6].

The *C. elegans* dauer larva thus represents an ideal model to investigate the mechanistic basis of a naturally-occurring mode of extended longevity. Biological ageing is defined as the gradual accumulation of cellular damage with age, governed by both environmental and genetic inputs [7]. Intensive research in gerontology has led to the emergence of a number of theories explaining the biological basis of ageing that are often mutually non-exclusive of one another; such as the

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century-old wear and tear theory, the Medawar's mutation accumulation theory that perceives ageing a "by-product" of natural selection, as well as the rate of living theory that links lifespan with the rate of oxygen consumption [7,8]. While there is no one prevailing theory of ageing, the oxidative stress theory currently represents one of the most well-received correlative theories of the ageing process [7]. The oxidative stress theory principally postulates that ageing is related to the deleterious effects of free radicals on cellular constituents including DNA, proteins as well as membrane lipids; and these reactive oxygen species are generated as byproducts of normal metabolic process integral to life, such as oxidative phosphorylation in the mitochondria [8].

Using a lipidomic approach comprising multiple reaction monitoring (MRM) transitions specific to distinct fatty acyl moieties in individual phospholipids, we demonstrated that in comparison to other developmental stages, the dauer larva contain a unique enrichment of polyunsaturated fatty acids (PUFAs) in their membrane phospholipids. We next sought to elucidate the changes in membrane lipid dynamics throughout the course of dauer endurance, and discovered that esterified PUFAs exhibited temporal accumulation during the early phases of dauer endurance (i.e. diapause), followed by sharp reduction prior to termination of diapause. Changes in esterified phospholipids were accompanied by concomitant increases in unbound PUFAs, as well as their corresponding downstream oxidized derivatives (collectively known as eicosanoids). Global phospholipidomics has therefore unveiled that PUFA sequestration in membrane phospholipids denotes an essential aspect of dauer dormancy, and a failure to upkeep membrane lipid homeostasis is associated with termination of dauer endurance.

2. Methods

2.1. Synchronization of C. elegans developmental stages

Wild type *C. elegans* strain Bristol (N2) was provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota, USA), which is funded by National Institute of Health Office of Research Infrastructure Programs. Nematodes were cultured and maintained at 20 °C on nematode growth medium (NGM) agar plates containing 200 µg/mL of streptomycin spotted with *Escherichia coli* OP50-1 as a food source, as previously described elsewhere [9]. To obtain synchronized developmental stages of *C. elegans*, gravid hermaphrodites were treated with sodium hypochlorite solution for fixed duration at defined volumetric ratios to obtain embryos. The embryos were then allowed to hatch on NGM agar plates overnight in the absence of food to obtain arrested first-stage larva (L1). L1 larva were washed off the plates and the density was estimated via counting the number of worms in diluted aliquots. The L1 larva were then deposited onto individual plates seeded with fixed concentration of OP-50-1 at a density of $10e^4$ larva per plate. The larva on individual plates were allowed to grow and develop into adults, and plates denoting each developmental stage was collected at specified time-points after the initiation of feeding [10]. Bacterial food source which may skew the *C. elegans* lipid profiles was removed via sucrose floatation prior to worm collection. Collected worms were frozen immediately in M9 buffer at −80 °C until lipid extraction.

2.2. Dauer induction and purification

N2 were grown in 500 mL of S-medium containing fixed concentration of bacterial food source for 10 days at 25 °C with constant agitation at 120 rpm. During this period of growth, food source was slowly depleted over time and overcrowding set in after repeated cycles of reproduction and multiplication, and dauers became plentiful at the end of the 10-day period. Worms were first isolated free of any remaining bacteria and tissue remnants by sucrose floatation. Next, dauers were purified by treating the isolated worms with 1% sodium dodecyl sulfate (SDS) for 15 min, followed by sucrose floatation and pelleting through 15% Ficoll [11]. These freshly isolated dauer population denotes Day 0 of our experimental time-point of dauer endurance. Purified dauers were plated on NGM plates at a density of $10e^4$ dauers per plate. Dauers were collected on representative time-points (Day 0, Day 7, Day 14 and Day 21) across the period of dauer endurance. Dauers were treated with 1% SDS for 15 min followed by sucrose floatation prior to collection to ensure that the collected samples were devoid of contaminating carcasses that may confound the lipid profiles.

2.3. Phospholipid extraction and HPLC-MS/MS analysis

Lipids were extracted from the *C. elegans* using a modified version of the Bligh and Dyer's method [12]. Briefly, frozen worm samples were first thawed on ice for 5 min, followed by the addition of chloroform:methanol 1:2 (v/v). Next, fixed amount of ceramic beads pre-cleaned with methanol to remove contaminating lipids were added, and the samples were homogenized on a bead ruptor (OMNI, USA) using a pre-optimized programme (speed =5 m/s, duration =8 s, pause =2 s, 2 cycles). Samples were then incubated at 4 °C for 1 h at 1500 rpm. At the end of the incubation, 400 µL of MilliQ water and 300 µL of chloroform were added. Samples were centrifuged at 12,000 rpm for 5 min at 4 °C, and the lower organic phase was extracted into a clean tube. The extraction procedure was repeated twice via addition of chloroform, and 50 µL of 1 M HCl was added at the last round of extraction. Extracted organic phases were pooled together into a single tube and dried under vacuum in the OH mode using the SpeedVac (Genevac, UK). Dried lipid extracts were stored at −80 °C until mass spectrometric analysis. $10e^4$ worms were collected per sample for analysis of phospholipidome.

Analytical methods were constructed based on high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) as previously described [13]. Lipidomic analyses were conducted on an Exion ultra-performance liquid chromatographic system (UPLC) coupled with Sciex 6500 Plus Qtrap (Sciex, USA). Qualitative deuterated lipid standards from LIPID MAPS were pre-corrected using suitable quantitative lipid standards from the same lipid class based on molar response prior to their use for quantitation. Separation of individual phospholipid classes of polar lipids by normal phase HPLC was carried out using a Phenomenex Luna 3 µm silica column (i.d. 150 × 2.0 mm) with the following conditions: mobile phase A (chloroform:methanol:ammonium hydroxide, 89.5:10:0.5), B (chloroform:methanol:ammonium hydroxide:water, 55:39:0.5:5.5). Multiple reaction monitoring (MRM) transitions specific to both head groups and fatty acid chains were set up for quantitative analysis of various phospholipids. Individual lipid species will be quantified by referencing to spiked internal standards, which included PC-14:0/14:0, PC34:1-d_{31}, LPC-d_4−26:0, PE-14:0/14:0, PE34:1-d_{31}, LPE-17:1, PS-14:0/14:0, PS-16:0/18:1-d_{31}, LPS-17:1, PA34:1-d_{31}, PA-17:0/17:0, LPA-17:0, PG34:1-d_{31}, PG-14:0/14:0, PI34:1-d_{31}, LPI-17:1 obtained from Avanti Polar Lipids and LIPIDS MAPS (Alabaster, AL, USA). Dioctanoyl phosphatidylinositol (PI, 16:0-PI) used for phosphatidylinositol quantitation was obtained from Echelon Biosciences, Inc. (Salt Lake City, UT, USA). For all LCMS analyses, individual peaks were examined and only peaks above the limit of quantitation and within the linearity range were included in the quantitation [14,15]. Lipidomic analyses were performed in triplicates for developmental stages and in quadruplicates for dauer endurance.

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